Correlation of Clinical Outcomes and Molecular Epidemiology of Epidemic Keratoconjunctivitis

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Abstract

Purpose: Adenoviral acute or epidemic keratoconjunctivitis (KC) is a common cause of ocular morbidity worldwide. The purpose of the study is to define the various species and types that exist in KC in correlation with the clinical presentations and outcomes with each species, and investigate the factors that predict poor clinical outcome in KC.

Methods: The clinical and molecular data from a randomized, controlled, masked trial for keratoconjunctivitis (NV-422 Phase IIB, NovaBay) was used in the study. In brief, 500 patients from United States, India, Brazil, and Sri Lanka with clinical diagnosis of KC and positive rapid testing for adenovirus (AdV) were included in the study. Clinical signs and symptoms and bilateral conjunctival swabs were obtained on day 1, 3, 6, 11, and 18. PCR was performed to detect and quantify AdV in all conjunctival samples.
The difference in composite scores of clinical signs and symptoms between day 1 and day 18 were evaluated by adenoviral species using ANOVA. Time to resolution of each symptom or sign were assessed by adenoviral species with Cox regression.

Results: Out of 500 patients, only 390 (78%) had evidence of AdV infection on PCR. Among AdV positive patients, AdV-D was most common (63%) but a total of 4 species and 21 different types of AdV were detected. The mean difference in composite scores of signs and symptoms between day 1 and 18 was significantly different by AdV species (p=0.003, p=0.0009, respectively). The hazard for resolution of lid edema (HR 0.41, adj.p=0.009), bulbar conjunctival injection (HR 0.55, adj.p=0.009), abnormal tear meniscus (HR 0.56, p=0.007), tearing (HR 0.54, adj p<0.006), and photophobia (HR 0.54, adj.p=0.04) were significantly lower in AdV-D group compared to non-D group. The improvement of visual acuity on day 18 was less in patients who started with better initial visual acuity, developed subepithelial infiltrates, and originated from countries other than US.

Conclusion: AdV-D infection related signs and symptoms are significantly more severe and less likely to resolve. Identifying patients infected AdV-D at early stage may have clinical implications in providing appropriate therapy and prognosis.

Introduction

Conjunctivitis is among the most common medical conditions, accounting for between 1 and 2% of all visits to primary care providers in the United States.[1] More than 4 million visits were made to physicians in the US for conjunctivitis in 2005,[2] and annual cost of diagnosis and treatment of conjunctivitis exceeds $430 million.[3] Conjunctivitis may be caused by bacteria, viruses, parasites or allergies, but viral causes are the most common. The most severe and common form of viral keratoconjunctivitis (KC) is epidemic keratoconjunctivitis (EKC), which has traditionally been associated with adenovirus (AdV) types 8, 19 (64), and 37.[4] Less frequently, types including 4, 53, 54, and 56 have also been reported in EKC. [5,6]

Because EKC is highly infectious, outbreaks in healthcare or community settings can last weeks to months.[7,8] For example, from 2008 to 2010, six isolated adenoviral epidemic outbreaks were reported in four states (Florida, Illinois, Minnesota, and New Jersey).[9] These outbreaks included more than 400 infected patients and forced a temporary closure of a neonatal intensive-care unit and several clinics. In the military, EKC can be equally costly. An outbreak at a US military base in the Phillipines in 1989 resulted in 2,603 cases and loss of 9,038 work-days, or almost 36 work-years in addition to incapacitating the base.[10] Even in a non-epidemic setting, adenoviral KC causes substantial economic burden in health-care due to inappropriate antibiotic uses and lost productivity in work or school.[2]

Despite the high morbidity and health-care costs associated with adenoviral KC, the diagnosis remains clinical. Previous studies have reported many different adenoviral species and types involved in keratoconjunctivitis such as AdV 2, 3, 4, 7, 8, 19, 37, and 54.[11] However, whether the clinical outcomes in each adenoviral species or type differ has not been studied. In addition, no specific treatment exists for AdV KC and no prognostic factors- either host or

pathogen related, have been defined, thus current management in KC consists of supportive measures only.

While most patients have no lasting visual sequela from EKC, a subset will develop subepithelial infiltrates (SEIs). These are considered pathognomonic for EKC[12] and have been associated with long-term vision loss in past studies;[13,14] in such patients a therapeutic intervention may be beneficial. At present, whether additional adenoviral species or types that are not associated with EKC also predispose to SEIs are unknown.

Recently, a large, randomized, controlled, masked trial of a candidate therapeutic agent for acute/epidemic keratoconjunctivitis (NV-422 Phase IIB, NovaBay) recruited 500 patients with clinical signs and symptoms consistent with EKC and evidence of AdV using an ELISA-based rapid screening test (Adeno Plus, Rapid Pathogen Screening Inc., Saratosa, FL). This was a pivotal FDA licensure trial for auriclosene, an aganoside antiviral (clinicaltrials.gov NCT01877694). The drug did not demonstrate efficacy, thus the collected data provides us with a rich dataset of natural history of EKC. To our knowledge, this is the largest clinical study of adenovirus-related keratoconjunctivitis as well as the largest repository of clinical samples ever collected in this disease. Using this well-characterized cohort, we sought to define the various species and types that exist in KC, correlate the clinical presentations and outcomes with each species, and investigate the factors that predict poor clinical outcome in keratoconjunctivitis.

Methods

Patients and Procedures. Patients were recruited at 58 ophthalmology centers in United States, Brazil, Sri Lanka, and India. The inclusion criteria included: 1) age 18 or older (except in the US where lower age limit was 1 year); 2) a recent history of URI or conjunctivitis symptoms, contact with infected person, or recent visit to eye care provider; 3) evidence of AdV using an ELISA-based rapid screening test (Adeno Plus, Rapid Pathogen Screening Inc., Saratosa, FL); and 4) at least 2 of the following 9 new onset signs acutely (less than 3 days) prior to enrollment including: bulbar conjunctival injection, conjunctival chemosis, follicular and/or papillary conjunctivitis, lid edema and/or lid erythema and/or ptosis, preauricular adenopathy, tarsal membranes or pseudomembranes, diffuse or focal keratitis, or bilateral involvement. Patients were excluded if any history of significant "greenish-yellow discharge," significant lid matting, seasonal ocular allergies with recent onset of moderate to severe itching, recent use of new eye drops, contact lens use, dry eyes requiring treatment, severe pain requiring narcotics or patching, and presence of SEIs at presentation.

All patients were evaluated on day 1, 3, 6, 11, and 18. The visit on day 42 was optional. Bilateral lower conjunctival swabs were collected on first five visits (day 1 to day 18 visits) and full ophthalmologic exams were performed. Visual acuity (VA) was measured with Early Treatment Diabetic Retinopathy Study (ETDRS) charts and converted to logMAR visual acuity. The following 10 clinical signs were assessed on each visit (day 1, 3, 6, 8, 11, 42): lymphadenopathy, lid edema, lid erythema, bulbar conjunctival injection, palpebral conjunctival hyperemia, conjunctival discharge/lid crusting, tear meniscus, corneal fluorescein staining, tear break-up time (TBUT), and the presence of subepithelial infiltrates. Six subjective ocular symptoms were assessed on every visit: the presence of blurry vision, foreign body sensation, tearing, itching, burning, and photophobia. Each sign and symptom was scored based on the

pre-specified scoring criteria as either 0,1 (absent, present) or 0,1,2,3 (absent, mild, moderate, severe).

Laboratory methods. Genomic DNA was isolated from Copan dry swab samples of the conjunctiva using NucliSENS easyMag (bioMerieux, Inc.) as per protocol. The adenoviral load (copies/swab) was measured by quantitative polymerase chain reaction (qPCR) on all specimens using the following universal primers and probes: TACATGCATCGCCGG, TACATGCATATCGCCGG, GGTCTTACATGCACATCTCGGG, CSGTGGTCACATCRTGGGT, CACGGGCACAAAACGCA, AGGCTGAAGTAGGTATCGGTGG, CCGGGTCTGGTGCTCGGTG, CCGGGTCTGGTGCAGT, CAGGACGCCTCGGAGTA, CCACGGACACCTACTTCACCCTGGG, and standards. The qPCR was performed in ABI 7500 FAST instrument using methods for single well and stored standard curve. Specific viral species and types were determined by sequencing of 400-500 base pairs (bp) of the AdV hexon gene.

Whole genome sequencing (WGS) was performed in selected AdV-negative samples (convenience sampling) using Illumina Nextera XT DNA library prep kit per manufacturer's protocol. Using the extracted genomic DNA, the samples were prepared using Illumina Nextera XT DNA library prep kit (Illumina, Inc. San Diego, CA) per manufacturer's protocol. In brief, libraries were created from a total of 1ng/ul of DNA per sample, which was tagmented, cleaned, and amplified using the provided index adapters. The resulting libraries were normalized and 2-8 uL of final products per sample was sequenced using Illumina Myseq machine.

Statistical analyses. The primary outcome of the study was the difference in composite scores of clinical signs between day 1 and day 18 by adenoviral species. Secondary outcomes included the time to resolution of each sign and symptoms in patients with AdV-D vs. the rest of the species (AdV-non D), the severity of clinical signs at different time points, the severity of clinical symptoms throughout the course, and the risk factors of SEI development and overall visual loss. Power calculation was performed based on the primary outcome to evaluate the

resolution of clinical signs in AdV-D vs. the rest of the species. Based on the data from our preliminary analysis, we assumed the composite score to have a standard deviation of about 21 points. With 316 patients with AdV-D and 74 patients with other species, we anticipated 84% power to detect a 1.75-point difference in the sum of scores between two groups. Normality of data was checked with qnorm plot. All analyses were performed with STATA 13.0 (Statacorp LP, College Station, TX) or R (http://www.rproject.org).

The details of our analysis methods are the following:

1) Descriptive Analyses

The data from the worse seeing eye on day 1 were included in the analysis of patients with bilateral involvement. If the visual acuity was the same on both eyes, then the study eye was randomly selected using a pseudorandom number generator. For comparison of categorical variables at baseline, we used Fisher's exact test and logistic regressions. Analysis of variance (ANOVA) was used for comparison of continuous variables at baseline.

2) Clinical Data Analyses

First, the composite scores of clinical signs and symptoms were analyzed with ANOVA. Second, time to resolution was assessed with Cox regression. Only patients who had any sign or symptom of a score greater than zero at baseline were included. Resolution was defined as a score of zero. The reference group in the survival analysis was AdV- non D group and was compared to either AdV-D or AdV-negative group. Multiple comparisons were adjusted using Bonferroni correction: p-values were multiplied by either 6 or 9 due to multiple symptoms and signs analyzed in the survival analyses, respectively.

3) Visual Outcome and SEI Development Analyses

The change in visual acuity (in ETDRS letters) was compared using univariate then multivariate linear regression analyses. Covariates chosen *a priori* for the multivariate linear regression model were: initial visual acuity, age, country of origin, and SEI development. The association

between SEI development and other risk factors were assessed with a logistic regression model.

Results

Patients were recruited from US (n=111), India (200), Sri Lanka (84), and Brazil (103), and randomized to auriclosene or vehicle arm at a 1:1 ratio. A total of 428 patients (86%) completed 18 day follow up. Mean age was 35 (range 1 to 90) and 290 were men (58%) (**Table 1**). Despite the requirement for positive result on Rapid Pathogen Screening test as an entry criterion, subsequent hexon sequencing and PCR found evidence of AdV in 390 (78%) patients. The distribution of adenoviral species, types, and adenoviral negativity differed significantly by geographic locations (p<0.001) (**Figure 1,2**). AdV-D was the predominant species in all sites except for US. Forty-four percent of the samples from US were AdV-negative (**Table 2**).

Demographics of the Patients and Initial Viral Load by Adenoviral Species. The mean age did not vary between the patients with AdV-negative keratoconjunctivitis or various AdV species (ANOVA, p=0.25). More women were affected than men in AdV-E but not in other species (58% vs. 25% to 40%, p=0.04) (**Table 2**). The initial viral load was similar between each species except for AdV-C, which appeared to have lower viral load than the rest of the groups; however, only 4 patients were included in this group (**Table 2**).

Distribution of Adenoviral Diversity and Adenoviral Negative Keratoconjunctivitis. Among adenoviral positive patients, AdV-D was most common (63%) and 37% of samples yielded other species (AdV-B, AdV-C, or AdV-E, in aggregate 15% of samples). Of the AdV-D samples, 262 (83%) were sequenced as type 8, while 9 (3%) were type 19 and 5 (1.5%) were

type 37. More than 10% of AdV-D samples represented other types. Overall, 4 species and 21 different types of AdV were detected.

To confirm the unexpected PCR sequencing results on AdV-negative samples, whole genome sequencing was performed in 16 conveniently selected AdV-negative specimens. There was no detectable adenovirus in 16/16 samples validating the PCR results.

Clinical Signs of Adenoviral Positive and Negative Keratoconjunctivitis. The mean composite scores of clinical signs on day 1 did not differ significantly between AdV species or AdV-negative keratoconjunctivitis (p=0.09). The mean sum ranged from 4.8 to 7.0 among different species and AdV-negative KC (**Table 3**). However, by day 3, the mean composite scores were significantly different between AdV species and AdV-negative KC with a lower score in AdV-negative KC (3.7) than AdV-positive group (weighted average all species 5.5) (ANOVA, p=0.0002). By day 6, all AdV species and AdV-negative KC showed decreasing trend in clinical scores. However, the AdV-D group retained relatively high score (4.4) compared to the rest of AdV species (weighted average in AdV-nonD group, 1.5). By day 11, all groups had mean scores <1 except for the group infected with AdV-D (2.0), and this pattern continued on day 18.

The mean composite scores of clinical signs on day 18 were significantly different in the group with AdV-D vs. the rest of species as shown in Figure 2. Except for the AdV-D group, the mean sum returned to baseline and remained stable by day 11 (**Figure 3a**). By ANOVA, the mean difference in composite scores of signs (defined as the difference in composite scores on day 18 and day 1) was significantly different by adenoviral species (p=0.003) (**Table 4**).

Time to Resolution of Clinical Signs, Survival Analysis. On day 1, the frequency of several signs varied substantially in patients infected with AdV-D, AdV-non D, and AdV-negative: lid edema (62.7% AdV-D, 66.0% AdV-non D, 55.5% AdV-negative), lid erythema

(37.0%, 51.4%, 40.9%), abnormal tear meniscus (44.1%, 47.3%, 31.8%) discharge (40.2%, 50.0%, 38.2%), palpebral conjunctival hyperemia (37.0%, 51.4%, 40.9%), and corneal staining (11.1%, 14.9%, 21.1%) (**Figure 4**).

The hazard for resolution of the following signs were significantly lower in patients infected with AdV-D compared to the group with AdV-non D: lid edema (HR 0.41, 95%CI 0.29-0.58, p=<0.001 adj. p=0.009), bulbar conjunctival injection (HR 0.55, 95%CI 0.41-0.75, p<0.001, adj. p=0.009), lid erythema (HR 0.64, 95%CI 0.44-0.95, p= 0.03, adj. p=0.27), abnormal tear meniscus (HR 0.56, 95%CI 0.37-0.85, p=0.007, adj. p=0.06), and discharge (HR 0.64, 95%CI 0.43-0.94, p=0.02, adj. p=0.18) (**Figure 5**). Multiplicity was adjusted by multiplying p-value by 9, the number of signs. The hazard ratio for resolving palpebral conjunctival hyperemia (HR 0.7, 95%CI 0.43-1.03, p=0.07), corneal staining (HR 1.11, 95%CI 0.52-2.38, p=0.79), lymphadenopathy (HR 0.94, 95%CI 0.55-1.6, p=0.82), and abnormal TBUT (HR 1.92, 95%CI 0.69-5.35, p=0.22) were not significantly different in patients with AdV-D vs. AdV-non D.

The hazard of resolution of clinical signs did not differ significantly between the AdV-non D and AdV-negative group: lid edema (HR 1.1, 95%CI 0.74-1.62, p=0.64), bulbar conjunctival injection (HR 0.91, 95%CI 0.64-1.3, p=0.62), lid erythema (HR 1.04, 95%CI 0.67-1.63, p=0.85), abnormal tear meniscus (HR 0.95, 95%CI 0.58-1.57, p=0.85), discharge (HR 1.11, 95%CI 0.71-1.75, p=0.64), palpebral conjunctival hyperemia (HR 1.0, 95%CI 0.64-1.57, p=0.99), corneal staining (HR 1.71, 95%CI 0.77-3.83, p=0.19), lymphadenopathy (HR 1.27, 95%CI 0.66-2.43, p=0.47), and abnormal TBUT (HR 2.25, 95%CI 0.74-6.84, p=0.15).

Clinical Symptoms of Adenoviral Positive and Negative Keratoconjunctivitis. The mean composite scores of clinical symptoms on day 1 and 3 did not differ significantly throughout the AdV species and AdV-negative group. However, by day 6, the mean composite scores of symptoms (mean 3.8, range 0 to 4.0) varied significantly between the with the highest score in patient with AdV-D (4.0) and the lowest in AdV-C(0) (**Table 3**). The mean composite

score in AdV-D group were 2.5 while the rest of the species had scores less than 2 on day 11 (2.5 vs. 0.2 to 1.4, p<0.0001) and day 18 (2.1 vs. 0.3 to 1.1, p=0.03) (**Figure 3b**). By ANOVA, the mean difference in composite scores of symptoms (defined as the difference in composite scores on day 18 and day 1) were significantly different by species (p=0.0009) (**Table 4**).

Time to Resolution of Clinical Symptoms, Survival Analysis. On day 1, the frequency of several symptoms varied substantially in patients infected with AdV-D, AdV-non D, and AdV-negatives: photophobia (31.3% in AdV-D, 37.8% in AdV-non D, 43.1% in AdV-negative), burning (48.8%, 50.7%, 54.9%) and foreign body sensation (61.1%, 70.3%, 56.0%) (**Figure 4**).

The hazard for resolution of the following symptoms were lower in patients infected with AdV-D compared to the group with AdV-non D: tearing (HR 0.54, 95%CI 0.40-0.75, p<0.001, adj.p<0.006), photophobia (HR 0.54, 95%CI 0.35-0.85, p=0.007, adj.p=0.04), burning (HR 0.64, 95%CI 0.44-0.93, p=0.02 adj. p=0.12), foreign body sensation (HR 0.68, 95%CI 0.49-0.93, p=0.02, adj.p= 0.12), and blurry vision (HR 0.61,95%CI 0.37-1, p=0.05, adj.p=0.45) (**Figure 5**). Multiplicity was adjusted by multiplying p-value by 6, the number of symptoms. No significant difference in hazard ratio of resolution time with itching (HR 0.89, 95%CI 0.62-1.28, p=0.52) was found between AdV-D and AdV-non D groups.

The hazard of resolution of the following clinical symptoms did not differ significantly between the AdV-non D and AdV-negative group except for photophobia (HR 0.6, 95%CI 0.37-0.99, p=0.05, adjusted p=0.30), tearing (HR 0.84, 95%CI 0.58-1.2, p=0.33), burning (HR 0.84, 95%CI 0.55-1.28, p=0.41), foreign body sensation (HR 0.78, 95%CI 0.52-1.15, p=0.21), itching (HR 0.9, 95%CI 0.59-1.37, p=0.61), and blurry vision (HR 0.85, 95%CI 0.49-1.47, p=0.56).

Visual Outcomes of Keratoconjunctivitis and Risk Factors. We computed change in visual acuity (VA day 18- VA day 1) during the observation period and refer to it as an

improvement measure. A positive value in this measure meant that patients gained VA on day 18 compared to baseline, while a negative value meant that patients lost vision. A substantial number of patients (N=104, 10%) had worsening of visual acuity over the course of adenoviral infection. However, the mean improvement in vision was a gain of 1.9 ETDRS letters (SD 8.0, range -30.0 to 35.0). The mean ETDRS visual acuity on day 1 was 92.0 letters (SD 8.5, range 50.0 to 115.0) and on day 18 was 93.5 letters (SD 9.0, range 50.0 to 113.0). 328 patients (77%) presented with visual acuity (VA) better or equal to 20/40 (85 ETDRS letters) while 100 patients (23%) had initial VA worse than 20/40 on day 1. 28 out of the 328 (9%) who started with vision better than or equal to 20/40 on day 1 worsened to less than 20/40 on day 18 (Figure 6, upper left quadrant); conversely, 55 out of 100 (55%) patients whose initial vision was worse than 20/40 reported improvement on day 18 (Figure 6, lower right quadrant).

By univariate linear regression analyses, the mean improvement in visual acuity was 0.4 ETDRS letters lower per 1 higher initial visual acuity letter ($p<0.001$), indicating reduced improvement or possibly worsening of visual acuity. In comparison to the patients from the US (mean VA change=3.6), the improvement in visual acuity was 1.9 ETDRS letters lower in patients from India (mean change=1.8, $p=0.06$), 2.9 letters lower in patients from Brazil (mean change=0.7, $p=0.01$), and 2.7 letters lower in patients from Sri Lanka (mean change=0.9, $p=0.02$), indicating reduced improvement relative to reference (**Table 5**). Patients who developed SEI had a lower VA improvement by 1.4 ETDRS letters relative to those who did not, but this difference did not reach statistical significance ($p=0.07$).

For those who had same age, country of origin, and SEI development status, the improvement in visual acuity was 0.4 ETDRS letters lower per one higher initial visual acuity letter (95%CI -0.5 to -0.3, $p<0.001$). For the patients who had the same initial visual acuity, country of origin, and SEI status, the change in VA was 0.1 letters lower per each year of age (95%CI -0.1 to -0.03, $p=0.002$) indicating reduced improvement in older patients. For those who had the same initial visual acuity, age, and SEI development status, the improvement in VA was

1.5 letters lower in patients from India, (95%CI -3.3 to 0.3, p=0.09), 2.3 lower in patients from Brazil (95%CI -4.4 to -0.1, p=0.04), and 2.5 lower in patients from Sri Lanka (95%CI -4.6 to -0.4, p=0.02) compared to the patients from the US. For those who had the same initial visual acuity, age and country of origin, the patients who developed SEI had 1.0 letter lower improvement than those who did not develop SEI (95% CI -2.4 to 0.4, p=0.16).

SEI development. Next we evaluated the factors associated with SEI development at any time over observation by univariate logistic regression. The odds of developing SEI was higher (OR=3.7) in AdV-D infection compared to AdV-non D (95%CI 2.2-6.2, p<0.001); in contrast, lower viral load at presentation was protective, OR=0.09 per log of initial viral load (95%CI 004-0.1, p=0.001) (**Table 6**). The treatment with auriclosene or placebo was not significant in this analysis for protecting or causing SEIs.

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Discussion

Our results highlight several important findings. First, unexpected diversity exists in adenoviral species and types in adenoviral keratoconjunctivitis. Second, a fair proportion of patients suffer from prolonged visual loss from adenoviral keratoconjunctivitis, even when the causative viral type is not usually associated with epidemic keratoconjunctivitis. Third, clinical sign and symptom severity correlates with adenoviral species. The disease course is much prolonged in patients with AdV-D compared to those with other species. Fourth, the risk of visual loss or SEI development is associated with both host and pathogen factors. Patients infected with AdV-D, in particular type 8, were at a significantly increased risk of developing visual sequelae. Lastly, approximately 20% of presumed adenoviral KC failed to show any evidence of adenovirus by state-of-the arts molecular techniques.

While a few studies have characterized molecular diversity of AdV keratoconjunctivitis,[11,15] studies on the disease course of different AdV species are much more limited. In 1983, Darougar et al. showed 21 different serotypes of 700 AdV KC specimens from Moorfileds Eye Hospital between 1973 and 1978.[15] In this series, 92% of infections were related to serotypes 3, 4, 7, 8, 10,15, and 19, and outbreaks were associated with AdV-B (type 3, 7), AdV-C (type 10, 15), and AdV-E (type 4). In a study of 98 consecutive patients with adenoviral infection diagnosed from conjunctival scrapings, serotype 3, 7, and 8 were most common. (86%, 84 out of 98).[16] In this study, male to female ratio was 2:1, unlike our study, which had no gender preponderance. The mean duration of conjunctivitis across all serotypes was 20 days, and the most severe keratoconjunctivitis was related to AdV-D, serotype 8. The authors concluded the AdV 11 (species B) as the type causing the shortest disease course and the AdV 5 (species C) the longest, but there were only 1 and 3 patients in each serotype group, respectively. Similar to our study, SEI was found in multiple serotypes including AdV 3, 4, 5, 7, and 8 infections. Recently, Butt and Chodosh reported 25.9% of 54 patients suffered chronic symptomatic keratitis with persistent SEI for more than 45 days following adenoviral keratoconjunctivitis.[13] One patient in this study required intermittent treatment for persistent SEIs for more than 2 years. However, the specific species or types of infections were not mentioned.

Despite the common conception that a pink eye occurs mainly in children, our study did not support that the children were the main reservoir of AdV. Only 15.9% of (18 out of 113) subjects were under age 18 in our US cohort, which had included children >1 year of age. This frequency is similar to Darougar et al's finding in which only 15% of patients were under 20 years in a review of 700 patient records.[15]

A large proportion of adenoviral negative KC was unexpected. However, the lack of AdV in patients with suspected AdV infection has been documented in.a study of 73 eyes from 65 patients in Saudi Arabia showed that only 65.7% were positive for AdV with immunochromatography tests.[5] Similarly, 60% were positive for AdV by PCR on 75 swabs from 66 patients with clinical presentation of conjunctivitis in Brazil between 2004 and 2007.[17] Thus, clinical exam alone is not a sensitive test in diagnosing AdV keratoconjunctivitis. To improve detection of AdV, Rapid Pathogen Screening test (RPS) was developed in 2006, an ELISA based test that detects a common epitope of the hexon protein of the adenovirus.[18] Previous studies have suggested that RPS detects all known molecular types of AdV, reaching up to 100% sensitivity and specificity.[19,20]

What is surprising of AdV-negative group in our study is that all subjects were positive for AdV entry by RPS, but the hexon sequencing and qualitative PCR of all samples found evidence of AdV in only 78% of the patients. As the sensitivity of PCR was ~100 copies/swab, this suggests the RPS likely was cross-reacting with a non-adenoviral protein epitope in these subjects. In addition, we considered the possibility of misclassification from non-infectious KC. However, patients were excluded if any history of previous allergic conjunctivitis or any chronic symptoms, and only the patients with new onset symptoms acutely (less than 3 days) were recruited. Furthermore, we found no reduction in the presence of symptoms or signs in AdV-negative group compared to the AdV-positive group to suggest a non-infectious etiology. Remarkably, 40 patients (36%) with adenoviral-negative KC developed SEIs during the course of infection.

To ensure the absence of adenoviral DNA in these samples, WGS was performed in 16 selected AdV-negative specimens. Results confirmed that there is no detectable adenovirus DNA in any of these samples, and no other known pathogenic viral or bacterial DNA was found.

Interestingly, torque teno virus (TTV), recently described in association with culture-negative endophthalmitis,[21] was found more frequently in AdV-negative (43%) than AdV-positive (13%) samples (OR 4.9, p=0.10); however, given that we find this virus in ~50% of normal subject conjunctival swabs (data not shown), we doubt that this virus is pathogenic on the eye surface. Possibilities for causative agents in AdV-negative subjects include RNA viruses, or small DNA viruses missed by WGS.

Even though SEIs are considered pathognomonic of EKC, the incidence of SEI was highly variable in our study. Interestingly, SEI formation was observed in a total of 14 AdV types (n=256) and in adenoviral-negative group (n=40). Given the variability of SEI incidence, SEI may be associated with additional host factors or viral molecular factors that are not captured fully with PCR alone. A recent study has demonstrated the genomic variability within type 8,[22] suggesting that the PCR sequencing highly underestimates the genetic variance of AdV. Future studies in molecular variants of AdV with additional molecular technique may provide insights into SEI development.

Clinical outcomes in KC are likely a function of both host and pathogen factors. The initial viral load was a risk factor, suggesting an important role of host ocular immunity; the finding that AdV-D types have higher likelihood of SEI development demonstrates the importance of pathogen-specific factors. However, given the SEI development in adenoviral-negative group and in multiple adenoviral strains, more complex pathogen-specific factors likely exist. Thus, uncharacterized adenoviral molecular variants or additional pathogens may play a role in the pathogenesis of SEI. What about the fact that viral load was a risk factor – is that a virus or a host factor – to me, suggests the role of ocular immunity.

Our study has several limitations. Even though patients were recruited if they developed symptoms acutely within 3 days, study subjects were likely presenting at different times in their infection's course. The distribution of species significantly varied by country of origin, and the visual outcome was associated with the country of origin, making this a confounder. Each country likely had variable access to care, patient populations, and treating eye-care providers. From the US, more patients with less severe signs and symptoms may have been recruited than from other countries due to better access to eye care. The US was the only site that recruited children and their data may have skewed the visual outcome results. However, when we analyzed the subgroup of children (n=19), they were less likely to test positive for AdV-D and develop SEI, supporting the overall conclusion that the visual outcome is associated with adenoviral genome.

Our data was collected during a period of 18 days. A significant amount of patients infected with AdV-D did not recover fully by day 18, thus the average duration of the disease course in AdV-D could not be fully studied. However, the study provides evidence that patients infected with other species reach near full resolution of all symptoms and signs around day 11.

Despite the limitations, our study has real world applications. Kuo et al. reported that only 4% of (22 of 518) hospital employees were found positive for AdV conjunctivitis based on real-time PCR.[23] The authors recommended a 14-day furlough in all patients who were positive for AdV on PCR. However, our study shows that the patients infected with AdV-non D are significantly more likely to have faster symptom and sign resolutions compared to the group with AdV-D. Thus, understanding the clinical course of each species may be helpful in setting appropriate policies in hospitals or other institutions and minimizing lost productivity. Additionally, given a higher likelihood of SEI development in patients infected with AdV-D, closer

follow up and more aggressive treatment[13,24] (i.e. topical corticosteroid, steroid-sparing or nonsteroidal anti-inflammatory agents) may be indicated in this subset of patients.

In summary, adenoviral keratoconjunctivitis is a common source of visual morbidity. Given that AdV-D disease course significantly differs from that of other species, identifying patients infected AdV-D early on will have clinical implications in providing appropriate therapy and prognosis. Further studies in adenoviral-negative keratoconjunctivitis will likely modify current diagnosis and management standards.

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